critical period in the rabbit during which administration of thalidomide is effective is not yet defined. It must be, however, very close to the times at which implantation, metamerism, and differentiation of the heart and adjacent vessels are nearing completion (8–9 days pc). This could account for the interesting syndrome of effects. It would also account for the interracial differences in syndrome and in number of defects, since there are fewer soft tissue anomalies in the OS and M strains than in III. This indicates the importance of knowledge of relative residual vulnerability to spontaneous malformation with reference to time and localization in strains used in drug testing. With one exception all of these animals were treated at the same embryonic age. The observed strain differences may indicate differential maturation of organs and tissues as the critical element determining localized vulnerability. Tabulation and analysis of control litters shows that treatment previous to current pregnancy has no significant residual effect on succeeding litters in all except the sternebral variation (reduced or absent 5th or 6th sternebrae) in strain III. A certain proportion of the abnormalities found in control populations, with one exception, fall in areas known from previous growth studies[9] to be vulnerable to retardation and thus have genetic origin. When the treated data are weighted for strain specific differences in vulnerability which cannot be due to thalidomide there remain highly significant strain differences in response to the drug[14].

Zusammenfassung. Thalidomid wurde in Gelatinkapseln peroral (500 mg/Tag/Kaninchen) an 30 gravide Kaninchen vom 6. bis 11. Tag nach Konzeption verabreicht. Von 109 klassifizierbaren Jungen waren nur 4% normal: 77 überlebten, 9 waren tot und 23 wurden abortiert; 20 weitere waren teilweise resorbiert und deshalb unklassifizierbar. Die drei genetisch verschiedenen Kaninchenstämme des Versuchs wiesen signifikante Unterschiede auf in Typus, relativer Zahl und Verteilung der Missbildungen.

P. B. Sawin, D. Crary,
R. R. Fox, and H. M. Wuest

Jackson Laboratory, Bar Harbor (Maine) and Sloan-Kettering Institute for Cancer Research, New York City (USA), June 17, 1965.

[14] This investigation was supported in part by Public Health Service research grant CA-00281 from the National Cancer Institute and by an allocation from Public Health Service General Research Support Grant No. 1 SO1 FR-05545-01 to the Jackson Laboratory, Bar Harbor (Maine).

Antibody Production by Blood Leucocytes

Introduction. Little is still known of the internal mechanisms of the cell which result in antibody formation, but there is considerable evidence for the presence of antibody-forming cells within the architecture of lymphoid organs, such as spleen and lymph nodes (Gowans and McGregor[1], Humphrey and White[2]). Production of antibody can also occur locally in almost any tissue provided antigen is present. These facts have been established by ablation of organs, by irradiation, by damage of lymphoid organs and by the extraction of antibody from tissues; much additional information was also obtained by transplantation of tissues from immunized animals to normal inbred or irradiated recipients.

It is the purpose of this communication to consider recent evidence for antibody-producing cells in yet another location, namely in lymph and blood. Gowans and McGregor[1] have summarized the data which leave no doubt that antibody-producing cells can be found in the circulation. Some of the recent studies in our laboratories (Hulliger and Sorkin[3,4], Landy et al.[5]) attest by quite separate means to the antibody-producing capacity of peripheral blood leucocytes. Some implications of these findings and the possibilities they raise for future work will be considered.

Methods. The experimental situations we investigated were (a) rabbits hyperimmunized with human serum injected intravenously, blood leucocytes being obtained for test 4 days after a booster injection, and (b) rabbits given a single intravenous injection of 5 μg of *Salmonella enteritidis* somatic polysaccharide, blood leucocytes being taken for test at daily intervals thereafter. In (a) de novo synthesis of antibody in vitro was demonstrated by incubation of 40–80 · 10^6 nucleated blood leucocytes with C^{14}-amino acids for 3 h and the incorporation into specific antibody was measured by the radioactivity present in the antigen-antibody precipitate produced by carrier human serum albumin-rabbit anti-HSA. In (b) the production of specific antibody was shown by a modification of the technique of localized hemolysis in gel (Jerne et al.[6]) in which 5–20 · 10^6 washed blood leucocytes in Eagle's medium plus agar were mixed with sheep erythrocytes that had been coated with *S. enteritidis* polysaccharide, the mixture poured into petri plates and allowed to solidify, incubated, and the plates overlaid with guinea-pig complement. The resultant zones of hemolysis (plaques) around individual leucocytes were specific for *S. enteritidis* polysaccharide in that plating the same leucocytes on normal sheep red cells, or on red cells coated with an immunologically unrelated somatic polysaccharide yielded no plaque formation.

Results. Twenty hyperimmunized rabbits were examined for antibody production by splenic and peripheral blood cells and appreciable antibody synthesis by blood leucocytes was demonstrated in 12 of the animals (Hulliger and Sorkin[3,4]). Some illustrative data are given in Figure 1.

[1] J. L. Gowans and D. D. McGregor, Progr. Allergy *9*, 1 (1965).
[2] J. H. Humphrey and R. G. White, in *Immunology for Students of Medicine* (Blackwell Scientific Publications, Oxford 1963), p. 135.
[3] L. Hulliger and E. Sorkin, Nature *198*, 299 (1963).
[4] L. Hulliger and E. Sorkin, Immunology *9*, in press (1965).
[5] M. Landy, R. P. Sanderson, M. T. Bernstein, and A. L. Jackson, Nature *204*, 1320 (1964).
[6] N. K. Jerne, A. A. Nordin, and C. Henry, in *Cell-Bound Antibodies* (Eds., B. Amos and H. Koprowski; The Wistar Institute Press, Philadelphia 1963), p. 109.

Whereas the control values for normal blood leucocytes ranged from 165 to 394 CPM, those for the immunized animals were 800 to 4700 CPM. In each of these reactors the values for antibody production by spleen cells were considerably higher than those for peripheral cells by a factor of 2 to 8 fold. Morphologic study of the cell population in spleen, lymph nodes and peripheral blood of the reactive immunized rabbits revealed a high proportion of plasma cells and plasma blasts (up to 43% in spleen, 15% in nodes and as much as 9% in blood); controls, on the other hand, showed few if any such cells in these sites. The aforementioned values were the highest obtained among the 12 reactors of the 20 immunized animals. Other reacting animals yielded lesser numbers of these cells; the values ranged down to approximately 3 times that found in the controls.

A total of 72 rabbits that received a systemic stimulus of 5 μg of *S. enteritidis* polysaccharide were studied for the production of antibody by peripheral blood leucocytes as measured by the technique of localized hemolysis in gel; for comparative purposes parallel tests were also made on spleen cells. In this work animals were sacrificed at various intervals after the immunizing injection in order to ascertain when plaque-forming cells (PFC) made their initial appearance, attained their maximum number, and the time when they no longer were evident (LANDY et al.[7]). Summary data on the numbers of PFC in blood as compared with those in spleen during the first week of the immune response are represented graphically in Figure 2.

It was found that small numbers of PFC were present in spleen as early as 14–18 h; after 48 h the number increased exponentially to reach a peak of approximately 2100 PFC per 10^6 cells at day 5. Thereafter the number of PFC decreased with approximately equal rapidity, so that by day 14–20 their number had plateaued at approximately 20 PFC per 10^6 splenic cells and this level remained unaltered for at least 2 months. As regards blood leucocytes, no PFC have been found in non-immunized controls (tests were made on $40 \cdot 10^6$ leucocytes). However, as early as 2 days after the immunizing injection, significant numbers of PFC were present in the blood of some animals, and on succeeding days the numbers of PFC increased and a higher proportion of the animals yielded peripheral blood PFC. Mean values for groups of 5–9 rabbits were 35 PFC/10^6 leucocytes at day 3, 170 at day 4, 65 at day 5 and 30 at day 6. From day 7 on no PFC were encountered in the blood of 17 animals.

The aforementioned investigations in our respective laboratories have dealt with experimental situations which differ in many ways. For one thing, the antigens employed were strikingly dissimilar, i.e. a relatively low molecular weight serum protein as opposed to a high molecular weight polysaccharide complex. Moreover the former is innocuous while the latter is toxic as well as antigenic. Secondly, the immunization procedures represent the extremes utilized by the immunologist; viz. a prolonged series of injections of protein, followed after a rest period by a booster injection, as compared with a single injection of polysaccharide. Thirdly, the quantity of antigen administered was of the order of 300 *mg* of protein as opposed to 5 μg of polysaccharide. Fourth, the immunization regimens are known to lead to the synthesis of different molecular forms of antibody. Repetitive stimuli with soluble proteins yield mainly 7S antibody (BAUER et al.[8]), whereas a single small dose of this polysaccharide has been shown to evoke a pure 19 response (WEIDANZ et al.[9]). Despite these many dissimilarities in each of the numerous factors involved in determining the character of the immune response, both experimenta situations gave rise to the appearance of antibody-forming cells in the peripheral circulation. Consequently it seems a reasonable assumption that the appearance of these cells in blood is hardly fortuitous; rather it is more likely to represent a significant facet of the overall immune response.

General discussion. Finding antibody-forming cells in blood justifies posing the question whether such cells had actually been stimulated while in the peripheral circulation. Relevant to this issue is the body of recent work on the transformation of human peripheral lymphocytes in vitro by various means, including phytohemagglutinin, specific antigen, antileucocytic serum etc. The resultant blast cells have been reported as producing γ-globulin (HIRSCHHORN et al.[10], PARENTI et al.[11]). These investiga-

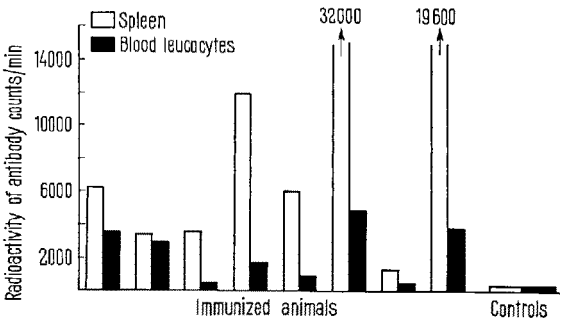

Fig. 1. Antibody production in vitro by blood leucocytes and spleen of rabbits hyperimmunized with HSA.

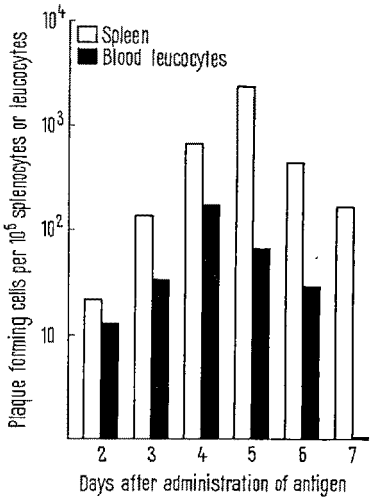

Fig. 2. Antibody production in vitro by spleen and blood leucocytes of rabbits after a single injection of Salmonella polysaccharide.

[7] M. LANDY, R. P. SANDERSON, and A. L. JACKSON, J. exp. Med. *122*, 483 (1965).
[8] D. C. BAUER, M. J. MATHIES, and A. B. STAVITSKY, J. exp. Med. *117*, 889 (1963).
[9] W. P. WEIDANZ, A. L. JACKSON, and M. LANDY, Proc. Soc. exp. Biol. Med. *116*, 832 (1964).
[10] K. HIRSCHHORN, F. BACH, R. L. KOLODNY, I. L. FIRSCHEIN, and N. HASHEM, Science *142*, 1185 (1963).
[11] F. PARENTI, P. L. FRANCESCHINI, G. FORTI, and R. CEPPELLINI, Atti Ass. genet. ital. *10*, in press.

tions thus provide indications that peripheral cells possess the basic potential to respond with the production of immunoglobulins.

It is noteworthy that with regard to the emergence of substantial numbers of antibody-forming cells in blood and their progressive increase in number, the events in peripheral blood mirror, to a large extent, the corresponding cellular changes in spleen. The scoring of animals with PFC in blood was 8/8 at 3 days, 4/5 at 4 days, 6/10 at 5 days and 3/7 at 6 days; the highest numbers of PFC were encountered between days 3 and 6. In this most active phase of the immune response 21/30 animals displayed very substantial numbers of PFC in peripheral blood. Whether the absence of discernible PFC in the remaining 9 animals has any special significance remains to be determined. The necessary processing of blood to remove erythrocytes and to concentrate leucocytes involves far more manipulation than the preparation of cell suspensions from spleen. The less uniform response in peripheral blood could conceivably involve technical factors, in which case it would be more apparent than real. This point merits further study.

In the system of the rabbits hyperimmunized with human serum albumin (HULLIGER and SORKIN[3,4]) morphological evidence indicated that at 4 days after the booster injection up to 9% of all leucocytes in blood seem to be committed to production of specific antibody as compared with up to 43% in spleen. In subsequent work with the same system it was found that at 2 days after a booster injection an even greater proportion of the peripheral leucocytes were morphologically of an antibody-producing character (HULLIGER and SORKIN[4]). The fact that in such hyperimmunized rabbits an astoundingly high proportion of the total number of leucocytes were now pyroninophilic cells constitutes no proof that these cells were necessarily stimulated in blood. This possibility cannot be excluded, however, in view of the work of PARENTI et al.[11] who reported that small lymphocytes from peripheral blood could be stimulated by phytohemagglutinin to transform directly without mitosis into pyroninophilic lymphocytes. However, it would seem to us rather remarkable that such large numbers of cells could have emerged from spleen alone[12]. The actual magnitude of the participation in the immune response by circulating cells is yet to be determined, but the data already in hand suggest that the contribution may be appreciable.

It is of course important to know the types of cells in blood responsible for this antibody production. In the case of the single antigenic stimulus, no information of consequence is available, inasmuch as the cell population studied, after removal of the granulocytes, consisted of typical small lymphocytes with a very few medium size lymphocytes. It is pointed out, however, that other morphologically distinctive cells could be present in small numbers and could easily have escaped detection since only about one cell in 10,000 was a plaque-forming cell.

Detailed morphologic studies were performed on the composition of the blood leucocytes in the hyperimmunized rabbits (HULLIGER and SORKIN[4]). There was found consistently a substantial increase in the number of pyroninophilic cells in blood at the time when antibody synthesis by blood leucocytes occurred. On the 4th day after a booster injection 12 of 20 rabbits studied showed these cells in blood, the number varied over a wide range, viz. 0.5 to 9%. Some of these pyroninophilic cells were plasmablasts and plasma cells while others also present in significant numbers were small pyroninophilic lymphocytes. Although this cell population was thus fairly diverse, it nonetheless consisted of cells which are in some way implicated in the process of antibody production.

The destination and fate of antibody-forming cells in blood poses still another problem. They could, of course, represent the means by which the capacity for antibody production is widely distributed. This presents an alternative possibility to the generally accepted view that distribution of antigen provides the means by which cells in various lymphoid sites are stimulated to antibody production. Conversely these may be cells which differ in important ways from those in spleen, for example, in that they are approaching the end of their antibody-producing phase. It may be that cells in this phase of their career are actively eliminated from lymphoid organs and relocate in other sites, perhaps finally in a morphologically different form.

At peak response, 4–5 days after injection of the polysaccharide, there are found approximately 2600 PFC/10^6 spleen cells and about 170 PFC/10^6 blood leucocytes, i.e. the concentration of PFC in blood leucocytes is roughly 1/16 that in spleen. The decline in numbers of PFC in spleen is stabilized at around 20 PFC/10^6 cells by day 18, whereas in blood by day 7 there seems to be an abrupt cessation of detectable PFC.

The appearance of PFC in peripheral blood of animals given a second injection of 5 μg of *S. enteritidis* polysaccharide 60 days later was studied in 22 rabbits. Mean values for the groups were as follows: 20 PFC/10^6 leucocytes at day 2 (3/3), 183 at day 3 (5/5) and 37 at day 4 (2/2); at day 5–11 none were evident in 8 rabbits. As manifested by earlier appearance, peak and disappearance of PFC it appears that the peripheral cell response to a second injection is speeded up significantly. The absence of PFC by day 7 after the first injection and by day 5 after a second injection is not likely to represent a 'dilution' effect since increments of as many as 40 million cells were plated and the method is known to be capable of measuring 10% of the PFC present in spleen from day 6–7 onward. It seems likely that by the time of peak response there has developed in spleen a heterologous population of cells, a significant proportion of which come into the blood. After the peak period, when PFC are no longer to be found in blood, significant numbers continue to be present in spleen. This could be interpreted as indicating that the cells in spleen at this time are different from those at peak response, in that the capacity to migrate out of the organ is now absent. Such 'non-migratory' cells may well have been present throughout, and only now are they discernable as the *sole* population. In cytological terms the 'fixed' or non-mobile PFC may be lymphoblasts or immunoblasts, while the mobile cells may be lymphocytes or plasma cells.

If antibody-producing cells are leaving fixed sites, an explanation is needed for this emigration. One of the possibilities which comes to mind could have its basis in the known trigger effect of antigen/antibody complexes on the formation of chemotactic factors. When antibody

[12] In this connection it should be noted that the number of mononuclear cells in the entire blood volume of the rabbit is roughly comparable to the number of monocytic cells in spleen. The calculations on which this statement is based are as follows: Blood volume is taken as 1/13 of the body weight and an adult 3 kg rabbit would thus have a blood volume of approximately 230 ml. With an average value of 5000 leucocytes per mm^3 the total is approximately $1.15 \cdot 10^9$ leucocytes of which about 1/3 or about $4 \cdot 10^8$ are mononuclear cells, which could be implicated in immunological events. The number of monocytic cells in the entire spleen was generally $3–5 \cdot 10^8$ as determined by chamber counts.

is formed by cells in the lymphoid organs and reacting with antigen, it is conceivable that under the influence of locally formed chemotactic factors certain types of cells would then migrate from the organ.

It was found that shortly after rabbits had been injected intravenously with 5 μg of somatic polysaccharide, a reduction in thymic cellularity began which, with time, became more pronounced until at day 5 (the interval of peak antibody response and maximum number of PFC in blood and in spleen) this organ then was, for all practical purposes, depleted of lymphocytes (LANDY et al.[13]). Thereafter the cells gradually reappeared and by day 20 the thymus was once again found to be a fully cellular apparently normal organ. Since the loss of thymic cellularity coincided with the progressive increase in the number of antibody-forming cells in spleen and in blood, the possibility of a temporal relationship between these two events was considered, i.e. that the cells escaping from the thymus accounted in part for the number of antibody-forming cells in blood measured by localized hemolysis. However, subsequent experiments on thymectomized adult rabbits showed that such animals were capable of responding to the polysaccharide stimulus with the usual number of PFC in both spleen and blood (LANDY and SANDERSON[14]).

The recent work of GOWANS on thoracic duct lymphocytes and the many investigations based on labelling with tritiated thymidine (FLOREY and GOWANS[15]), have shown that lymphocytes are capable of extensive migration via lymph, blood and lymphoid organs. The present work emphasizes that cells actually engaged in synthesis of antibody are capable of similar mobility; once in the circulation these cells could be transported to even the most distant sites. As regards the immunological properties attributed to lymphocytes, these have generally been considered to be capabilities *other* than the production of humoral antibody. Thoracic duct cells are commonly viewed as being a 'pure' population of lymphocytes; accordingly studies on the hyperimmunized rabbits have been extended to include cells from this source (HULLIGER and SORKIN[4]). Antibody production was found in thoracic duct cells of some of these animals and in addition pyroninophilic cells were demonstrated in significant numbers (as compared to controls). However, since pyroninophilic cells were sometimes encountered in thoracic duct lymph in our control animals these findings do not necessarily constitute evidence that lymphocytes per se can produce antibody.

The demonstration of immunologically active cells in sites other than those traditionally associated with the process of antibody synthesis should now perhaps be viewed with greater caution. The knowledge that cells producing antibody come into the circulation raises from a possibility to a certainty that at least some of these cells would reach and lodge in sites not generally associated with the process of antibody production. Thus the detection of antibody-producing cells by immunofluorescence, incorporation of labelled amino acid into immunoglobulin, autoradiography or by localized hemolysis need not be interpreted as evidence that this activity had actually arisen in locations not ordinarily associated with antibody production. For example, we have reported the finding of antibody-producing cells in thymus (LANDY et al.[13]) after a single intravenous injection of antigen. Furthermore, very considerable numbers of PFC have been found in the liver of rabbits given a single intravenous injection of polysaccharide (SANDERSON and LANDY[16]). One could of course believe that cells in these organs had been directly stimulated, but it seems to us more reasonable to consider the alternative probability that in the thymus there had occurred an infiltration of antibody-forming cells from other sites, and in the liver, because of the relatively enormous blood flow through this organ, antibody-forming cells in the circulation had somehow been trapped or occluded in this organ and local proliferation may have occurred. In this connection may be mentioned studies which suggest that antibody production within the liver (in response to ovalbumin and pneumococcal polysaccharide) is usually correlated with the presence of 'invading' cells forming scattered periportal granulomate leading to the conclusion that hepatic parenchymal cells do not synthesize antibody (HUMPHREY and WHITE[2]). In view of the findings presented here the detection of antibody-producing cells in unfamiliar locations might best be viewed in terms of the transport mechanism provided by the circulation and that additional evidence be sought before concluding that antibody production had been initiated in non-lymphoid tissues[17].

Zusammenfassung. Zirkulierende Blutleukocyten produzieren Antikörper nach mehrmaliger Injektion von Protein-Antigen oder einmaliger Verabreichung von Endotoxin. Ursprung und Bestimmungsort dieser Zellen werden diskutiert.

E. SORKIN and M. LANDY

Medizinische Abteilung, Schweizerisches Forschungsinstitut, Davos (Switzerland), and Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda (Maryland USA), April 23, 1965.

[13] M. LANDY, R. P. SANDERSON, M. T. BERNSTEIN, and E. M. LERNER, Science *147*, 1591 (1965).
[14] M. LANDY and R. P. SANDERSON, unpublished observations.
[15] H. W. FLOREY and J. L. GOWANS, in *General Pathology* (Ed., H. W. FLOREY; Lloyd-Luke Ltd., London 1962), p. 128.
[16] R. P. SANDERSON and M. LANDY, unpublished observations.
[17] This work was supported by the Swiss National Foundation for Scientific Research, Grant No. 2635.

CORRIGENDUM

G. W. BARNES, M. A. SULLIVAN, E. H. BEUTNER, and E. WITEBSKY: *In vitro and in vivo Interaction of Nuclear Antibodies with Corresponding Antigens*, Experientia *21*, fasc. 8, p. 485 (1965). The legend for the one and only Figure of the paper reads correctly as follows: 'Direct immunofluorescent staining of S.L.E. (PAT) liver smear with anti-human conjugate. × 500. Upper: Conjugate neutralized with human γ-globulin (Cohn Fr. II). Negative reaction. Lower: Active conjugate. Note nuclear and cytoplasmic staining.' The text-lines (p. 486) referring to this Figure read correctly as follows: 'A few of the stained cells in liver films, in addition showed a distinct nuclear reaction (Figure, lower).' and 'The serologic specificity of the immunofluorescent reaction was confirmed by negative results obtained with similar slides treated with γ-globulin-neutralized conjugate (Figure, upper).' The footnote of Table II reads correctly as follows '[a] Tested at an earlier date.'